Amendments to the Specification

Please replace the paragraph *beginning on page 1, line 8* with the following amended paragraph:

-The present invention is concerned with bubonic plague caused by *Yersinia ~~Pestis~~ pestis* and vaccines for treating same-.

Please replace the paragraph *beginning on page 2, line 3* with the following amended paragraph:

-Examples of the second category function to promote lethality following infection by the intravenous route, known to facilitate immediate transport of the bacteria to favored niches within visceral organs (4). Mutational loss of these lethal factors causes qualitative (intravenous 50% lethal dose > 10^7 bacteria) decreases in virulence. Included in this group are certain ca. 70 kb low calcium response or Lcr plasmid encoded proteins: V antigen (9, 27), others termed Yops (18, 19, 33, 47): YopE (23, 35, 43, 44, 47), YopH (3, 34, 44), and probably YpkA (13), as well as chromosomally encoded antigen 4 or pH ~~PH~~ 6 antigen (20) and possibly the murine exotoxin encoded by the ca. 100 kb Tox plasmid (32.). Considerable effort has been spent in study of the regulation, processing, and delivery of these proteins to host cells (2, 15, 26, 28, 30, 31, 35).--

Please replace the paragraph *beginning on page*

2, line 30 with the following amended paragraph:

-Plague vaccines have been identified in U.S. Patent No. 3,137,629. The patent describes a process for producing killed plague vaccines which immunizes mice and guinea pigs by growing *Pasteurella pestis*, killing the strain through mechanical action and solubilizing the extract in strong alkaline solution, and then preparing ~~parental~~ parenteral vaccine by reducing the pH value of the soluble *P. pestis* antigenic solution to a neutral pH.-

Please replace the paragraph *beginning on page 3, line 24* and continuing to *page 4, line 4* with the following amended paragraph:

-The invention is further concerned with a method of controlling the effects of *Y. pestis* in mammals comprising the steps ~~step~~ of:

a) providing a vaccine comprised of the protein encoded by the construct of Figure 1; and

b) treating a mammal in need thereof with an effective anti-*Y. pestis* amount of the vaccine.--

Please replace the paragraph *beginning on page 4, line 19* with the following amended paragraph:

-FIGURE 3 are immunoblots prepared with rabbit polyclonal anti-V ~~anti V~~ antigen (A) mouse monoclonal

anti-V ~~anti V~~ antigen 15A4.8 (B) and mouse monoclonal anti-V ~~anti V~~ antigen 3A4.1 (C) directed against whole cells of E. Coli containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKVE14 ~~pKE14~~ (lane 2);--

Please replace the paragraph *beginning on page 5, line 7* with the following amended paragraph:

-FIGURE 5 is a chart of antiserum for passive immunization against native V antigen, recombinant V antigen, recombinant ~~protent A-C antigen~~ protein A-V antigen fusion and recombinant protein A;-

Please replace the paragraph *beginning on page 5, line 11* with the following amended paragraph:

-FIGURE 6 are immunoblots prepared with rabbit anti-native V antigen (A) or mouse monoclonal 17A5.1 anti-V ~~anti V~~ antigen (B) directed against truncated protein A (PA) (lane 1), protein A-V antigen fusion peptide (PAV) (lane 2), PAV partially hydrolyzed by formic acid (lane 3), PAV partially hydrolyzed by formic acid and passed through the IgG Sepharose 6FF column (lane 4), whole Lcr^+ cells of *Yersinia pestis* KIM (lane 5), and whole $LCR^{-\cdot}$ cells of *Y. pestis* KIM (lane 6); A-V_d, V_o, V_d, and A indicate the positions of PAV, native V antigen (37 Kda), truncated V antigen (29.5 kDa), and truncated Protein A, respectively. Human γ-globulin was

used to block nonspecific reactions of monoclonal antibodies against IgG-binding domains of Protein A (26); and--

Please replace the paragraph *beginning on page 5, line 25* with the following amended paragraph:

-FIGURE 7 are immunoblots prepared with rabbit anti-protein A-V antigen fusion peptide (A) and anti-truncated protein A (B) directed against whole cells of *Escherichia coli* containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKVE14 ~~pKE14~~ (lane 2); Also shown are reactions against disrupted and centrifuged whole cells of *E. coli* (pKE14) (lane 3) and further fractionation of V antigen by chromatography on phenyl-Sepharose CL-4B (lane 4), DEAE (diethylaminoethyl) cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7), and a second passage on DEAE cellulose (lane 8).--

Please replace the paragraph *beginning on page 6, line 7* with the following amended paragraph:

-The medically significant yersiniae (*Yersinia pestis, Y. pseudotuberculosis,* and *Y. enterocolitica*) are known to share a ca. 70 kb low calcium response (Lcr) plasmid that mediates restriction of vegetative growth at 37°C in Ca^{2+}-deficient media while promoting selective

synthesis of virulence factors including V antigen. The latter, encoded by *lcrV* on the Lcr plasmid, is established as a 37 kDa protective antigen capable of undergoing possible autoproteolytic hydrolysis. In this study, *lcrV* of *Y. pestis* was cloned under control of the strong *tac* promoter into protease-deficient *Escherichia coli* BL21. The resulting recombinant V antigen, like native V antigen, underwent degradation during purification yielding major peptides of ca. 36,35,34 and 32 to 29 kDa. Rabbit γ-globulin raised against this mixture of cleavage products provided partial but significant protection against 10 minimal lethal doses (MLD) of the three species. To stabilize V antigen and facilitate its purification, plasmid pPAV13 was constructed so as to encode a fusion of *lcrV* and the structural gene for saphylococcal protein A (e.g. all but the first 67 N-terminal amino acids of V antigen and the signal sequence plus IgG binding domains but not cell-wall associated region of protein A). The resulting protein A-V antigen fusion peptide (PAV) could be purified to homogeneity in one step by IgG affinity chromatography and was found to be stable thereafter. Rabbit polyclonal γ-globulin directed against PAV provided substantial passive immunity against 10 MLD of *Y. pestis* and *Y. Pseudotuberculosis* but was ineffective against *Y. enterocolitica*.--

Please replace the paragraph *beginning on page 7, line 6* with the following amended paragraph:

-The vaccine as described herein is generally applied by ~~parental~~ <u>parenteral</u> administration to mammals in need thereof.-

Please replace the title *beginning on page 9, line 2*, with the following amended title.

-Characterization of Deletional Variants of HindIII Fragment From the *IcrGVH-yopBD* Operon of *Yersinia* <u>*pseudotuberculosis*</u> ~~Pseudotuberculosis~~ 995.--

Please replace the table *beginning on page 9, line 5*, with the following amended table.

Encoding Plasmid	Size of Fragment	Operon	Designation Of V Antigen	Size of V Antigen (kDa)
pBVP5	~3,500	~~1crgvh-ypoB~~ <u>1crGVHyopBD</u>	V_o	37.3
pBVP513D	2,184	1crGVH	V_o	37.3
pBVP53D	1,484	$1crGV_1$	V_1	31.5
pBVP514D	1,160	$1crGV_2$	V_2	19.3
pBVP515D	878	$1crGV_3$	V_3	8.5
pBVP58D	705	$1crGV_4$	V_4	2.0
pBVP55D	546	$1crG_1$	-	-

Please replace the paragraph *beginning on page 10, line 17* with the following amended paragraph:

-<u>Purification of Recombinant V Antigen</u>. Cells of *E. coli* BL21 (pKVE14) were grown in fermenters as

described previously (5) in medium containing 3% Sheffield NZ Amine, Type A (a pancreatic hydrolysate of casein which contains mixed amino acids and peptides and is used to facilitate the growth of bacteria) (Kraft, Inc., Memphis, Tenn.), 0.5% NaCl, 1% lactose, and ampicillin (100 µg/ml) at 37°C and harvested by centrifugation (10,000 x g for 15 min) at an optical density (620 nm) of about 1.2. After disruption in a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) and removal of insoluble matter by centrifugation (10,000 x g for 30 min), V antigen was subjected to purification by an established procedure (5). The method involved use of hydrophobic interaction chromatography with ~~Phenyl~~ phenyl-Sepharose CL-4B (Pharmacia), ion exchange chromatography with DEAE cellulose (Whatman Inc., Clifton, J.J.), gel filtration chromatography with Sephacryl S-300SF (trademark of Pharmacia Biotechnology Group for acrylic resin for chromatographic separation of proteins), and Bio-Gel HTP (trademark of Bio-Rad, Richmond, California, for calcium hydroxyapatite ~~(~~chromatography~~)~~). The original procedure was supplemented by a second chromatographic separation of DEAE cellulose (linear gradient from ~~0~~ 0.35 M NaCl) in order to remove high molecular weight material peculiar to *E. coli*.-

Please replace the paragraph *beginning on page 11, line 10* with the following amended paragraph:

-Preparation of PA and PAV. Cells of *E. coli* transformed with pPAV13 or PRIT5 were grown to late log phase at 37°C in Luria broth containing ampicillin (50µg/ml). Purification of these recombinant proteins was accomplished by affinity chromatography on IgG Sepharose 6FF (Pharmacia) according to directions supplied by the manufacturer. Briefly, the procedure involved harvesting the organisms by centrifugation (10,000 x *g* for 16 min) with resuspension at a ca. 10-fold increase in number in 0.01 M Tris.HCl, pH 8.0 (column buffer). Lysis was accomplished by addition of lysozyme (5 ~~Mg~~ mg/ml) and, after incubation for 1 h, further addition of Triton X-100 (trademark of ~~Aahm~~ Rohm & Haas Co. for a nonionic detergent comprised of octyl ~~phenopypolyethoxy~~ phenoxy polyethoxy ethanol having an HLB: 13.5_j (0.1%) whereupon incubation was continued for 3 to 4 h. After clarification by centrifugation (10,000 x *g* for 30 min), samples of 400 ml of the resulting enriched periplasm were passed through a column (10 x 100 mm) containing a 10 ml packed volume of affinity resin that selectively bound PA or PAV. After addition and elution of 10 void volumes of column buffer to remove contaminating matter, the recombinant proteins were eluted with 0.2 M acetic acid (ca. pH 3.4), immediately

frozen, and ~~the~~ then lyophilized. Resulting purified PA and PAV were then used directly for qualitative analysis and immunization.-

Please replace the paragraph *beginning on page 12, line 25* with the following amended paragraph:

-Rabbit polyclonal antisera was raised against PA and PAV with Freund's adjuvant as described previously (62). TiterMax™ adjuvant (~~Hunger's~~ Hunter's TiterMax #R-1, CytRx Corp., Norcross, Ga.) was used to immunize rabbits against recombinant V antigen plus its degradation products purified from *E. coli* BL21 (pKVE14). Antisera prepared against recombinant V antigen or fusion proteins were not absorbed with material from ~~Lcr~~ Lcr$^-$ bacteria although highly purified γ-globulin was isolated from these reagents as described previously (62). Antisera raised against V antigen purified from *Y. pestis* or E. coli BL21 (pKVE14) is termed anti-native V antigen or anti-recombinant V antigen, respectively.-

Please replace the paragraph *beginning on page 13, line 7* with the following amended paragraph:

-Immunoblotting. Alkaline phosphatase conjugated with anti-rabbit or anti-mouse IgG (~~Sigma~~ Sigma Chemical Co., St. Louis, Mo.) were used as secondary antibodies in immunoblotting by procedures

essentially identical to those already defined (51,52). In order to prevent nonspecific reactions of antibodies with PA and PAV, the nitrocellulose filter was first blocked with 5% fetal calf serum as usual and then incubated overnight in a solution of 1% normal human γ-globulin (Calbiochem, San Diego, Calif.). Human γ-globulin (0.5%) was also added to solutions of primary and secondary antibodies (26). In addition, Fc-specific anti-mouse IgG (A-1418, Sigma) was used as a secondary antibody during immunoblotting of fusion proteins and their derivatives with monoclonal antibodies.-

Please replace the paragraph *beginning on page 13, line 22* with the following amended paragraph:

-Passive Immunity. The ability of highly purified ~~Y~~ γ-globulin obtained from unabsorbed rabbit polyclonal antisera raised against recombinant V antigen, PA, and PAV to provide passive immunity was assayed by defined methods (40,62). Briefly, this procedure involved intravenous injection of 10 minimum lethal doses (MLD) of *Y. pestis* (10^2 bacteria), *Y. ~~Pseudotuberculosis~~ pseudotuberculosis* (10^2 bacteria), or *Y. enterocolitica* (10^3 bacteria) followed by intravenous administration of either 100 μg ~~or 500~~ or 500 μg of purified γ-globulins on postinfection days 1, 3, and 5.-

Please replace the paragraph *beginning on page 14, line 7* with the following amended paragraph:

-Degradation of Recombinant V Antigen. Recombinant plasmid pKVE14 containing the *1crGVH-ypoBD* operon of *Y. pestis* under control of the *tac* promoter was transferred into protease-deficient *E. ~~col~~ coli* BL21. After growth in fermenters, the bacteria were disrupted and the resulting extract was used to prepare nearly homogenous recombinant V antigen using a method established for Ca^{2+}-starved cells of *Y. pestis* (5). An additional step involving a second separation with DEAE cellulose was necessary to eliminate major higher molecular weight proteins present in *E. coli* cytoplasm.-

Please replace the paragraph *beginning on page 14, line 18* with the following amended paragraph:

-The initial specific activity of recombinant V antigen was almost 5-fold greater than that obtained from *Y. pestis* starved for Ca^{2+} (5). Nevertheless, significant loss of precipitin activity occurred during every step of purification (Table 2). This phenomenon, as judged by a silver-stained extended lane gel (Fig. 2), reflected gradual loss of the native 37 kDa form with emergence of ca. 36 kDa, 32 kDa, and possibly smaller peptides. Analysis by immunoblotting was undertaken to prove that these new peptides shared epitopes with and

thus arose from native V antigen. Use of rabbit polyclonal anti-native ~~V.~~ V Antigen (Fig. 3A) or mouse monoclonal antibody 15A4.8, directed against a centrally located epitope (Fig. 3B), demonstrated emergence of ca. 36, 35, and 34 kDa degradation products early during the course of purification with later appearance of a series of smaller fragments ranging from 32 to 29 kDa. The latter were not recognized by mouse monoclonal antibody 3A4.1 directed against an epitope located near the C-terminal end (Fig. 3C). These findings indicate that recombinant V antigen produced in protease-deficient *E. coli* BL21 undergoes ~~evidence~~ evident spontaneous degradation in a manner similar to that observed from native ~~V.~~ V antigen expressed in *Y. pestis* (5). Furthermore, patterns observed upon immunoblotting with monoclonal antibodies indicate that the C-terminal portion of V is involved in this process.-

Please replace the paragraph *beginning on page 17, line 1* with the following amended paragraph:

-Passive Immunity Mediated By Anti-recombinant V Antigen. A portion of the purified lot of recombinant V antigen described above was used to immunize rabbits. Immunoblots of the resulting unabsorbed antisera (Fig. 4B) and control absorbed anti-native V antigen (Fig. 4A) versus Ca^{2+}-starved whole yersiniae were identical

indicating that the reagent was monospecific. Both antisera were tested for ability to confer passive immunity against intravenous infection with yersiniae. As shown in Fig. 5, the control anti-native V antigen provided complete, partial, and insignificant protection against ~~y.~~ Y. *pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, respectively. Antirecombinant V antigen promoted a similar degree of passive immunity except that that directed against *Y. pestis* was not absolute.-

Please replace the paragraph *beginning on page 17, line 17* with the following amended paragraph:

-Characterization of PA and PAV. Additional constructions encoding truncated staphylococcal Protein A either alone or fused with V antigen (Fig. 1) were found, after transformation into *E. coli* BL21, to promote significant synthesis of PA and PAV, respectively as judged by intensity of the specific immunoblots described below. PA and PAV were purified in one step with IgG Sepharose 6FF and then analyzed by immunoblotting. Anti-native V antigen reacted nonspecifically with PA (Fig. 6A, lane 1) and both specifically and nonspecifically with PAV (Fig. 6A, lane 2). Proof that the salient peptides shown in lanes 2, 3, and 4 of Fig. 6A reacted specifically rather than nonspecifically with anti-native V antigen was obtained by blocking PA with human γ-

globulin and then immunoblotting with monoclonal anti-V antigen. This process prevented visualization of PA (Fig. 6B, lane1) thus demonstrating that the remaining detectable bands represent a specific interaction with an epitope of V antigen. Multiple bands appearing in samples of both PA and PAV (Fig. 6A, lanes 1, 2) reflect accumulation in the periplasm of *E. coli* BL21 of the synthesized PA domain in both native and degraded forms as described by others (16). To prove that the V antigen domain of the fusion protein was stable, a sample of purified PAV was hydrolyzed with 70% formic acid to cleave acid labile Asp-Pro sites defined in Fig. 1B, neutralized, and then applied to the affinity column. Essentially pure truncated V antigen (V_d) emerged immediately (Fig. 6, lane 4); the absence of multiple bands in this sample provides evidence for the stability of V antigen within PAV.

Please replace the paragraph *beginning on page 18, line 22* with the following amended paragraph:

-Passive Immunity Mediated By Anti-PAV. Preparations of homogenous γ-globulin were isolated from unabsorbed rabbit antisera raised against PA and PAV purified by affinity chromatography. Control Anti-V antigen (Fig. 4A and 4B) and anti-PAV (Fig. ~~5C~~ 4C), but not anti-PA (Fig. ~~5D~~ 4D), reacted with V antigens of all

three *Yersinia* sp. High molecular weight antigens (ca. 70 kDa) common to both Lcr^+ and Lcr^- yersiniae were recognized by both anti-PAV (Fig. 4C) and anti-PA (Fig. 4D). Anti-PAV (Fig. 7A) but not anti-PA (Fig. 7B) also identified the same degradation products of V antigen that were detected by the antisera characterized previously (Fig. 3). These findings verify that anti-PAV contains antibodies directed against epitopes unique to V antigen and provide further evidence that its degradation occurs at the C-terminal end.-

Please replace the paragraph *beginning on page 20* with the following amended paragraph:

-Concerns that these precautions, undertaken to assure monospecificity of anti-V antigen, had inadvertently introduced uncontrolled variables were largely eliminated by use of γ-globulin purified from antisera raised against highly purified V antigen cloned in *E. coli*. However, this process was also unsatisfactory due to the occurrence of marked degradation throughout the course of purification. As a result, only a fraction of the final product consisted of the 37 kDa primary *1crV* product. Although γ-globulin purified from unabsorbed antiserum raised against this mixture provided satisfactory passive immunity, yields of antigenic material were insufficient to permit widespread

immunization. The observation that cloned V antigen expressed in the ~~prototease~~ protease-deficient background of *E. coli* BL21, like native V antigen purified from *Y. pestis*, underwent marked degradation during purification further suggests but does not prove that this process is autocatalytic.-

Please replace the paragraph *beginning on page 20, line 24* with the following amended paragraph:

-Problems concerning specificity and degradation were resolved upon development of the fusion protein PAV that could be isolated at high yield as a homogenous stable protein in a single step. Antisera raised against PAV ~~was~~ were somewhat more effective in providing protection against *Y. pestis*, and especially *Y. pseudotuberculosis* than was anti-recombinant V antigen. This finding emphasizes that passive immunity mediated by anti-V antigen does not require interaction with N-terminal epitopes because the latter were absent in PAV. The independent observation that the N-terminal end of V antigen was poorly antigenic (37) is consistent with this conclusion.-

Please replace the paragraph *beginning on page 21, line 7* with the following amended paragraph:

-FIGURE 1. Scheme of construction of

recombinant plasmid of pPAV13 encoding staphylococcal protein A-V antigen fusion protein (PAV) (A) and characterization of PAV (B). Sites of restriction endonuclease attack are designated; Ap and Cm are locations of markers of resistance for ampicillin and chloramphenicol, respectively. Lac designates the position of *lacZ* which provides selection of recombinant plasmids in the vector pBluescript SK+. The genes *1crG, 1crV,* and *1crH* comprise a portion of the *1crGVH-yopBD* operon of ~~*yersinia*~~ *Yersinia pseudotuberculosis* 995 (38) and the designation Protein A is the truncated Protein A gene of *Staphylococcus aureus*. The dark arrows in A represent the hybrid gene encoding PAV shown in B to consist of the signal sequence (S), IgG-binding domains (E to B), the defective domain C' that has lost the ability to bind IgG, and truncated V antigen that has lost the first 67 amino acids of its N-terminal portion. Molecular weights in Kilodaltons are designated for each peptide arising after hydrolysis of the acid-labile Asp-Pro cleavage sites marked by arrowheads (60).-

Please replace the paragraph *beginning on page 21, line 28* with the following amended paragraph:

-FIGURE 2. Silver-stained 12.5% extended sodium dodecyl sulfate-polyacrylamide electrophoresis gel of whole cells of *Escherichia coli* BL21 containing the

vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKVE14 (lane 2). Whole cells of *E. coli* (pKVE14) were disrupted and ~~centrifugal~~ centrifuged to prepare a cell-free extract (lane 3) that was fractionated by chromatography on phenyl-Sepharose CL-4B (lane 4), DEAE cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7), and a second passage on DEAE cellulose (lane 8). Note the presence of V antigen in lanes 2 through 8 as a major peptide of 37 kDa.-

Please replace the paragraph *beginning on page 22, line 9* with the following amended paragraph:

-FIGURE 3. Immunoblots prepared with rabbit polyclonal anti-V antigen (A), mouse monoclonal anti-V antigen (15A4.8 (B), and mouse monoclonal anti-V antigen 3A4.1 (C) directed against whole cells of *Escherichia coli* containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid ~~pKE14~~ pKVE14 (lane 2). Also shown are reactions against disrupted and ~~centrifugal~~ centrifuged whole cells of *E. coli* (~~pKE14~~ pKVE14) (lane 3) and further fractionation of V antigen by chromatography on phenyl-Sepharose CL-4B (Lane 4), DEAE cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7) and a second passage on DEAE cellulose (lane 8).-

Please replace the paragraph *beginning on page 24, line 1* with the following amended paragraph:

-FIGURE 7. Immunoblots prepared with rabbit anti-protein A-V antigen fusion peptide (A) and anti-truncated protein A (B) directed against whole cells of *Escherichia coli* containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKE14 (lane 2). Also shown are reactions against disrupted and centrifuged whole cells of *E. coli* (~~pKE14~~ pKVE14) (lane 3) and further fractionation of V antigen by chromatography on phenyl-Sepharose CL-4B (lane 4), DEAE cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7), and a second passage on DEAE cellulose (lane 8).-